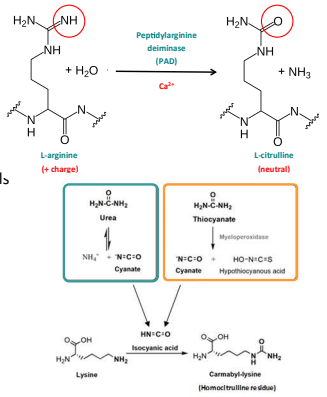
Vaccination stimulating post-translational modification specific Th1 responses repolarises the tumour environment to reduce suppressive LAP expressing T cells

Suha Atabani, Katherine Cook, Peter Symonds, Ian Daniels, Ruhul Choudhury, Alissa Wright, Anne Skinner, Victoria Brentville and Lindy Durrant.

Scancell Holdings plc, Oxford UK,

INTRODUCTION

- Cancer cells adopt resistance mechanisms to abrogate the immune responses and escape surveillance allowing tumour progression.
- Such mechanisms of resistance include recruitment of immunosuppressive cell types such as regulatory T cells (T regs) and increased activity of inhibitory pathways. T-regs can inhibit both naturally occurring or even therapeutically induced immune responses.
- T regs expressing cell surface TGFβ complexed to latency associated peptide (LAP) marks a population of CD4+ T regs increased in cancer and more suppressive than conventional Foxp3+ CD4+ Tregs [1, 2].
- Despite many suppressive mechanisms Th1 CD4 T cells have the capacity to influence the tumour environment through the release of pro-inflammatory cytokines such as IFNγ and TNFα as well as promotion of chemokine gradients that encourage infiltration of immune cells including antigen presenting cells (APCs), macrophages and CD8 T cells.
- CD4 responses to self-antigens are often attenuated.
- Cellular stress can lead to post-translational modification (PTM) of proteins that can be recognised by the immune system [3]. Such modifications include citrullination (cit) and carbamylation/homocitrullination (hcit).
- Peptidyl arginine deaminases (PADs) that mediate citrullination are activated by high levels of calcium in autophagosomes [3]. Homocitrullination is mediated by myeloperoxidase (MPO) released from MDSCs in tumours [6].
- Stressful conditions in the tumour microenvironment promote autophagy and lead to presentation of modified peptides on MHC class II. These MHC II presented modified peptides are targets for CD4 T cells. These T cells can be harnessed for tumour therapy [4-7].
- In the absence of inflammation, immunity is regulated. In the presence of inflammation, Th1 CD4 responses to modified self-antigens are stimulated.



Citrullination. A modification that occurs within stressed cells. Peptidylarginine deiminase (PADs) enzymes are activated and convert arginine to citrulline by altering the positively charge aldimine group (=NH) group of arginine to the neutrally charged ketone group (=O) of citrulline.

Carbamylation. A modification that is driven by increased isocyanic acid levels. This occurs physiologically due to the breakdown of Urea (A) or the actions of MPO enzyme on thiocyanate (B). MPO is produced by neutrophils and MDSCs. Carbamylation converts positively charged lysine to neutrally charged homocitrulline.

Table 1. Peptides used in the study

Antigen	Peptide coordinates	Sequence*	modification	abbreviation	Human or mouse
GRP78(BIP)	189-208	TIAGLNVM-cit-IINEPTAAAIA	citrulline	BIPcit	human
Vimentin	415-433	LPTFSSLNL-cit-ETNLESLPL	citrulline	V415cit	human
Vimentin	28-49	cit-SYVTTST-cit-TYSLGSAL-cit-PSTS	citrulline	V28cit	human
Alpha-enolase	241-260	VIGMDVAASEFY-cit-SGKYDLD	citrulline	E241cit	human
Nucleophosmin	266-285	AKFINYVKNCF-cit-MTDQEAIQ	citrulline	NPMcit	human
Cytokeratin 8	101-120	KFASFIDKV-cit-FLEQQNKMLE	citrulline	Cyto8cit	human
Aldolase	74-93	IGGVILFHETLYQ-hcit-ADDGRP	homocitrulline	A74hcit	human
Aldolase	140-157	hcit-DGADFA-hcit-WRCVL-hcit-IGEH	homocitrulline	A140hcit	human
Vimentin	116-135	NYID-hcit-VRFLEQQN-hcit-ILLAEL	homocitrulline	V116hcit	human
Alpha-Enolase	11-25	IFDS-cit-GNPTVEVDLY	citrulline	Eno11-25cit	mouse
Cytokeratin 8	8-26	KSYKSTSGP-cit-AFSS-cit-SFT	citrulline	Cyto8(8-26)cit	mouse
Glutamate receptor (NMDA 2B)	316-340	EPKSSCYNTHEK-cit-IYQSNMLN-cit-YLI	citrulline	GluRecep316-340cit	mouse

* cit = citrulline, hcit = homocitrulline

The T cell repertoire to some modified peptides is attenuated in lung and ovarian cancer patients compared to healthy donors.

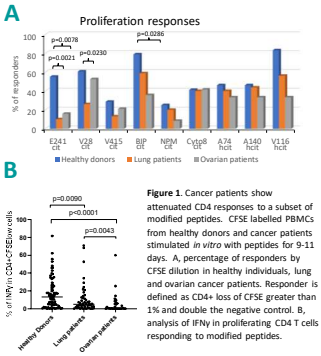


Figure 1. Cancer patients show attenuated CD4 responses to a subset of modified peptides. CFSE labelled PBMCs from healthy donors and cancer patients stimulated *in vitro* with peptides for 9-11 days. A, percentage of responders by CFSE dilution in healthy individuals, lung and ovarian cancer patients. Responder is defined as CD4+ loss of CFSE greater than 1% and double the negative control. B, analysis of IFNγ in proliferating CD4 T cells responding to modified peptides.

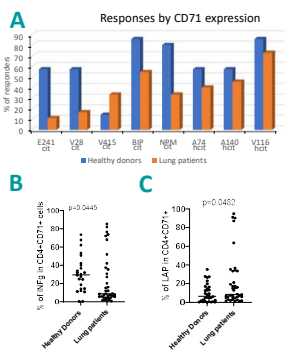


Figure 2. Cancer patients have higher LAP expression on CD4 T cells responding to modified peptides. PBMCs from healthy donors and cancer patients stimulated *in vitro* with peptides for 9-11 days and stained for CD71 expression. A, the percentage of responders by CD71 expression in healthy individuals and lung cancer patients. Responder is defined as greater than 1% and double the negative control. B, analysis of IFNγ in CD4+ CD71+ T cells. C, analysis of LAP expression on CD4+ CD71+ T cells

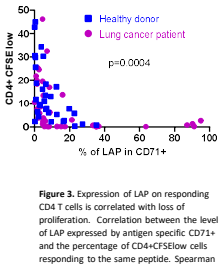


Figure 3. Expression of LAP on responding CD4 T cells is correlated with loss of proliferation. Correlation between the level of LAP expressed by antigen specific CD71+ and the percentage of CD4+CFSElow cells responding to the same peptide. Spearman correlation test was used for statistical analysis.

Expression of citrullinated vimentin in NSCLC is a good prognostic factor and absence of citrullinated vimentin combined with high LAP expression on TILs is a poor prognostic factor

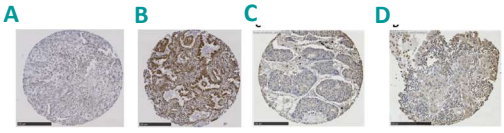


Figure 4. IHC staining of lung tumour cells for citrullinated vimentin and LAP in TILs. A: low citrullinated vimentin expressing tumour with H score zero and B: high citrullinated vimentin expressing tumour with H score 190. C: low LAP-TILs staining with H score of zero, D: is high LAP-TILs staining with H score of 20.

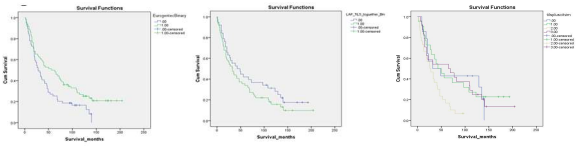


Figure 5. Kaplan-Meier plots for disease-specific survival for (A) cytoplasmic citrullinated vimentin where blue is low staining and green is high staining (p=0.016). (B) LAP-TILs where blue is low staining and green is high staining (p=0.232). (C) co-expression of LAP on TILs and citrullinated vimentin where blue are tumours with no expression of citrullinated vimentin and low LAP on TILs, purple are tumours with expression of citrullinated vimentin and had high LAP on TILs, green are tumours which expressed citrullinated vimentin and had low LAP on TILs and yellow are tumours which lacked expression of citrullinated vimentin and had high LAP on TILs (p=0.018).

Murine tumour models show infiltration of CD4+ LAP+ TILs and this can be modulated by peptide vaccination targeting modified epitopes

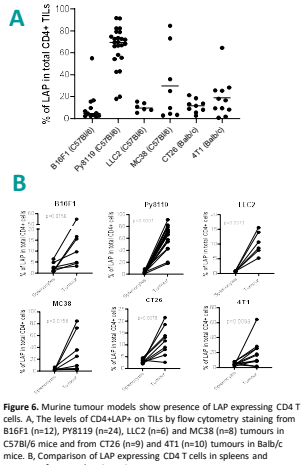


Figure 6. Murine tumour models show presence of LAP expressing CD4 T cells. A, The levels of CD4+LAP+ on TILs by flow cytometry staining from B16F1 (n=12), PY8119 (n=24), LLC2 (n=6) and MC38 (n=8) tumours in C57Bl/6 mice and from CT26 (n=9) and 4T1 (n=10) tumours in Balb/c mice. B, Comparison of LAP expressing CD4 T cells in spleens and tumours of tumour bearing mice.

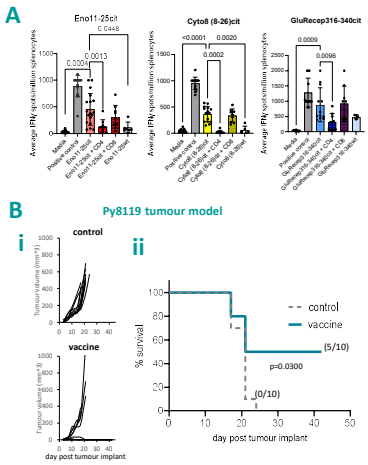
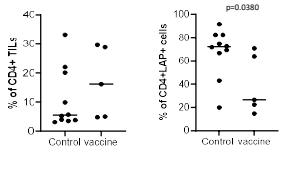


Figure 7. Citrullinated peptide vaccination promotes tumour therapy and modulates LAP expressing CD4 TILs. A, C57Bl/6 mice immunised via subcutaneous route with combination of 25μg each peptide with 5μg each of CpG and MPLA adjuvants on days 1,8 and 15 and peptide specific responses in splenocytes assessed by IFNγ ELISpot assay on day 21 to citrullinated peptides in the presence of CD4 or CD8 blocking antibodies (n>5). B, Tumour growth curves (i) and tumour free survival (ii) of C57Bl/6 mice implanted with Py8119 tumour on day 1 and immunized with a combination of citrullinated peptides in CpG and MPLA adjuvants on days 3, 7 and 10 (n=10). Numbers in brackets represent number of tumour free animals out of total. C, Py8119 tumours from control or vaccinated mice were analysed for CD4 infiltration (i) and percentage of LAP expressing CD4 TILs (ii) by flow cytometry.



CONCLUSIONS

- **PTM specific CD4 T cell responses escape central tolerance.**
- **Cancer patients show higher levels of CD4 T cells responding to modified peptides that express LAP, implying the immunosuppressive tumour environment polarises responses.**
- **Citrullinated vimentin affords a good survival prognosis in NSCLC, particularly with low LAP expression on TILs.**
- **Murine tumour models show LAP expressing CD4 TILs that can be reduced by stimulating Th1 responses to tumour expressed citrullinated antigens via vaccination which promotes tumour therapy.**

References:

1. Chen ML et al. Latency-associated peptide identifies a novel CD4+CD25+ regulatory T cell subset with TGFbeta-mediated function and enhanced suppression of experimental autoimmune encephalomyelitis. J Immunol. 2008; 180:7327–7337
2. Scurr M et al. Highly prevalent colorectal cancer-infiltrating LAP(+) Foxp3(−) T cells exhibit more potent immunosuppressive activity than Foxp3(+) regulatory T cells. Mucosal Immunol. 2014; 7:428–439
3. Ireland JM, Unanue ER. Autophagy in antigen presenting cells results in presentation of citrullinated peptides to CD4 T cells. J Exp Med. 2011 Dec 19;208(13):2625-32.
4. Brentville VA, et al. Citrullinated vimentin presented on MHC-II in tumor cells is a target for CD4+ T cell-mediated antitumor immunity. Cancer Research 2016 Feb 1;76(3):548-60
5. Cook K et al. Citrullinated alpha-enolase is an effective target for anti-cancer immunity. Oncoimmunology, 2018. 7(2): p. e1390642.
6. Cook KW et al. Homocitrullination of lysine residues mediated by myeloid-derived suppressor cells in the tumor environment is a target for cancer immunotherapy. Journal for immunotherapy of cancer 2021;9:e001910
7. Symonds P et al. Citrullinated Epitopes Identified on Tumour MHC Class II by Peptide Elution Stimulate Both Regulatory and Th1 Responses and Require Careful Selection for Optimal Anti-Tumour Responses. Front Immunol, 2021. 12: p. 764462